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WO 91/13075 A2 US 5137806 A
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(58) Field of Search

INT CL⁶ C12Q 1/68

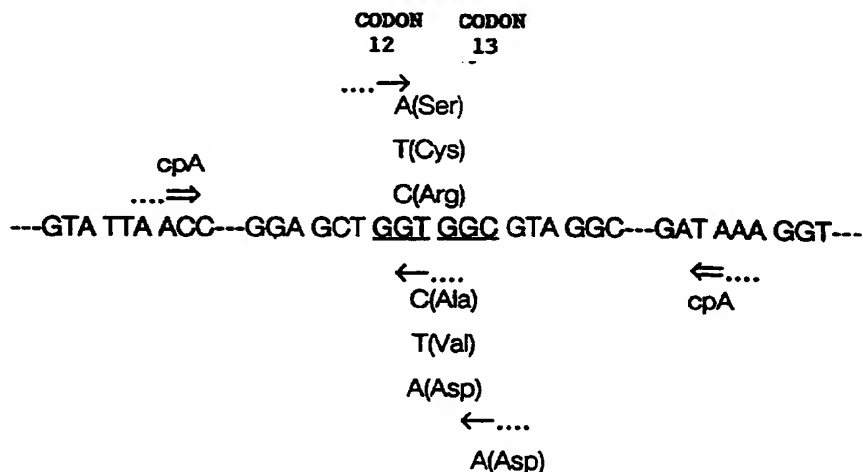
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(54) Abstract Title

Diagnostic assay for cancer

(57) A diagnostic assay for the detection of K-ras mutations in cancer. The method comprises contacting a test sample of nucleic acid with a diagnostic primer for a K-ras mutation in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when a K-ras mutation is present in the sample; and detecting the presence or absence of a diagnostic primer extension product. Diagnostic primers for seven K-ras point mutations are provided. Also included is a diagnostic kit in which one or more diagnostic primers are conveniently packaged with appropriate nucleotide triphosphates, polymerase, buffer and instructions for use. Amplification from the diagnostic primer may be by ARMS or ALEX techniques, and may be monitored by real time detection of product.

FIGURE 1



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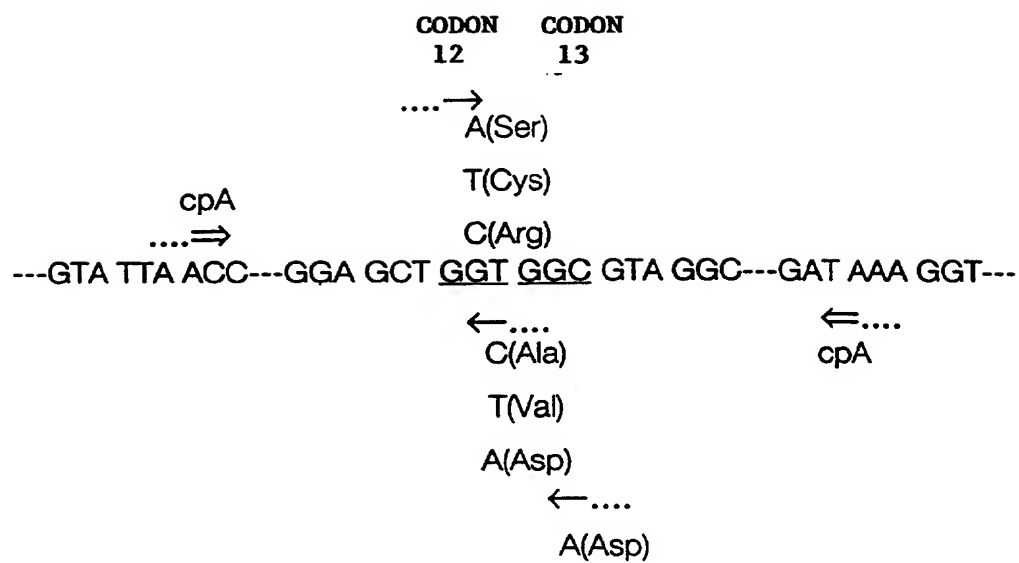
FIGURE 1

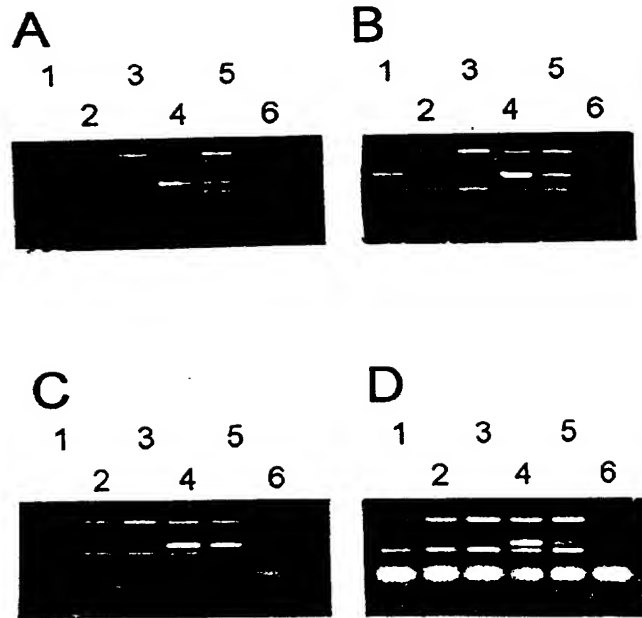
FIGURE 2

FIGURE 3

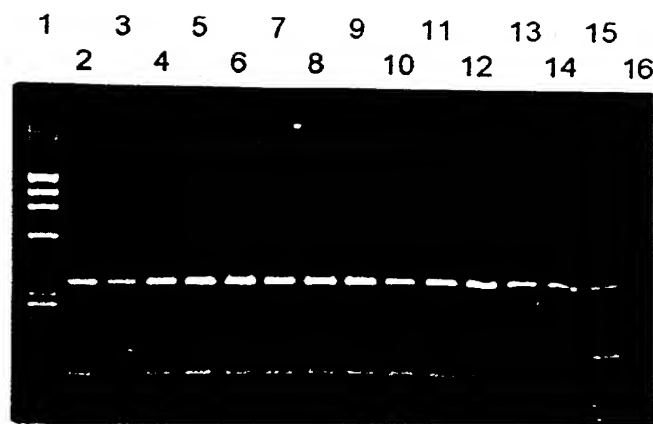
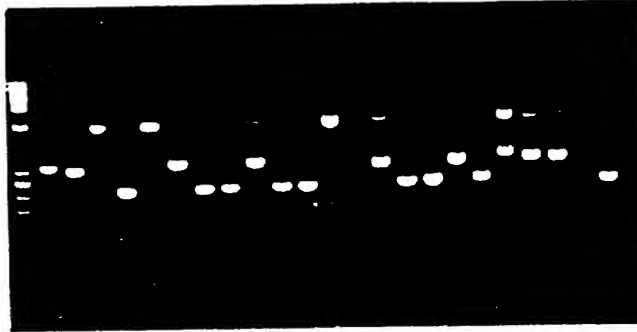


FIGURE 4

1 3 5 7 9 11 13 15 17 19 21 23
2 4 6 8 10 12 14 16 18 20 22 24



25 27 29 31 33 35 37 39 41 43 45 47
26 28 30 32 34 36 38 40 42 44 46 48

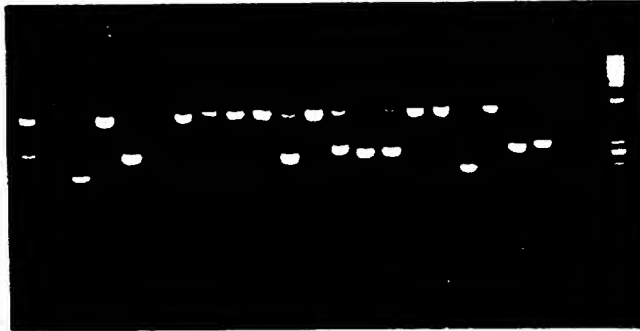


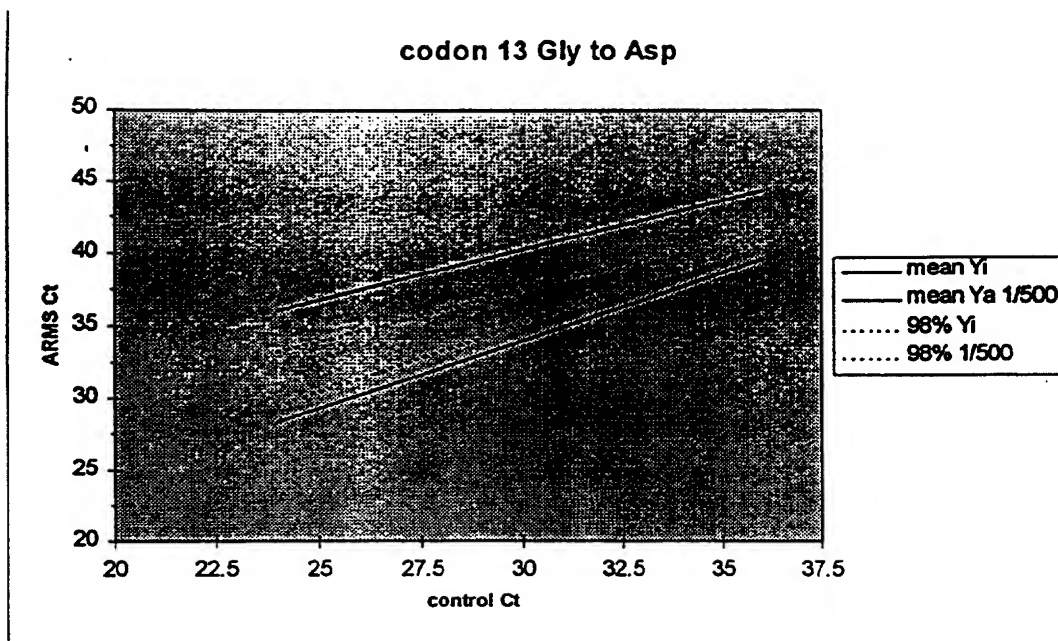
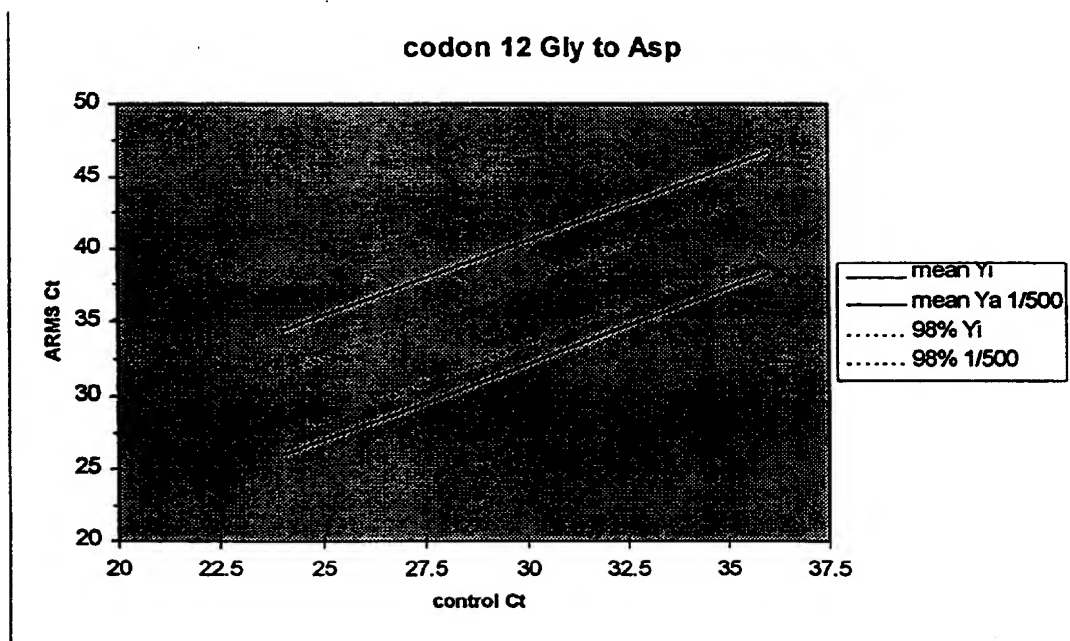
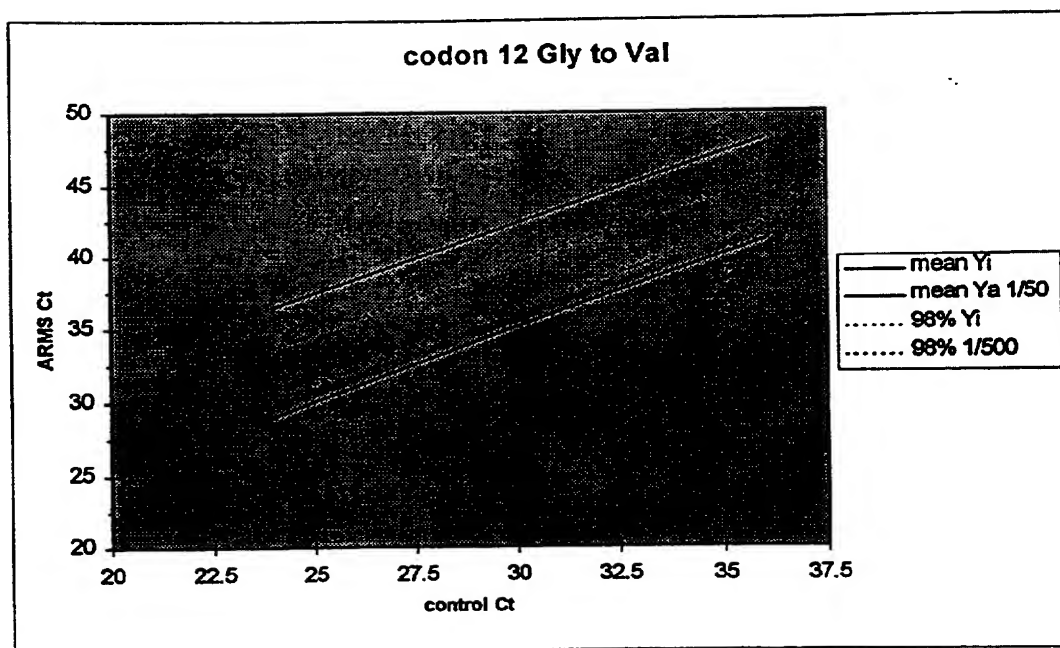
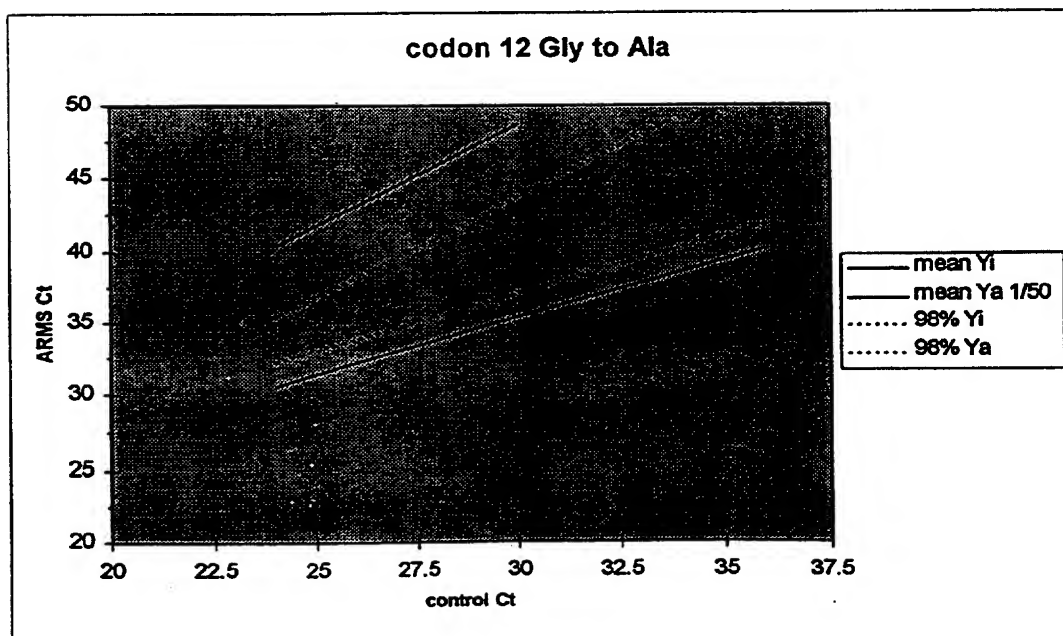
FIGURE 5**5a****5b**

FIGURE 5 (Cont'd)

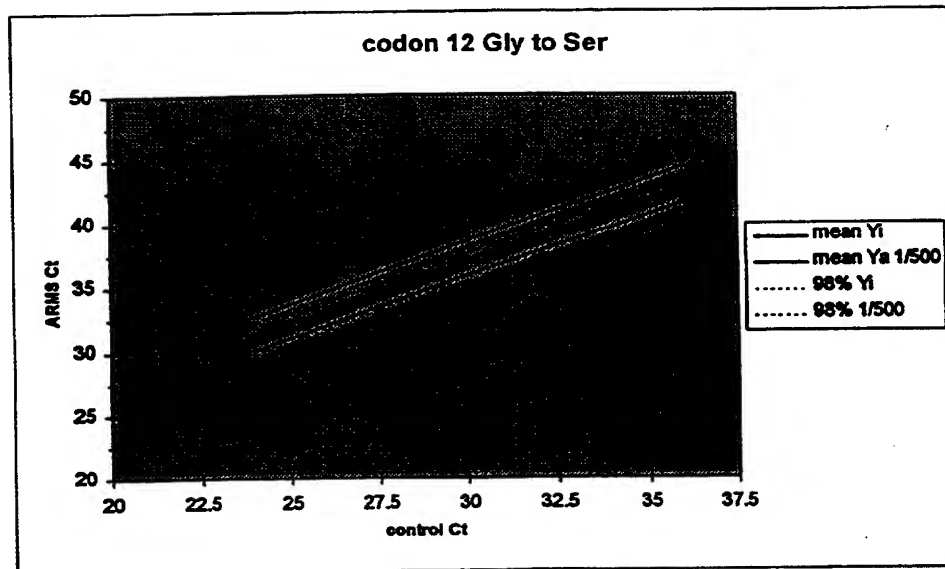
5c



5d

FIGURE 5 (Cont'd)

5e



5f

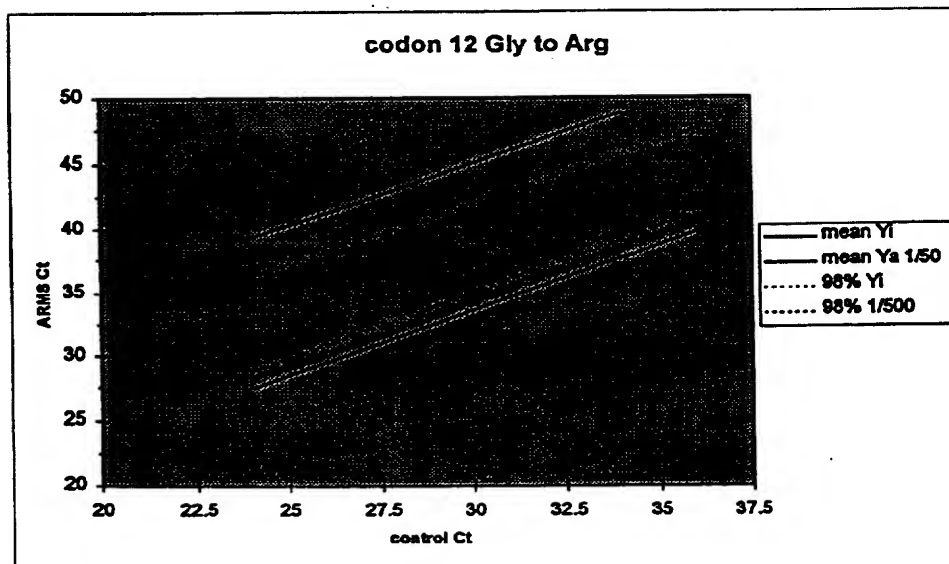
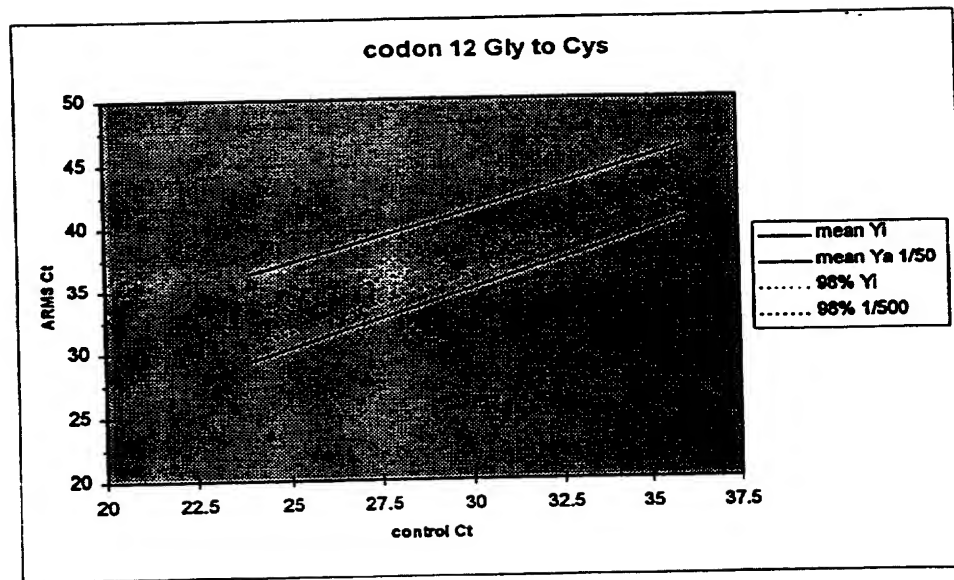


FIGURE 5 (Cont'd)

5g

ASSAY

This invention relates to a diagnostic method for the detection of K-*ras* mutations in cancer, particularly colorectal cancer, using the amplification refractory mutation system, (ARMS).

The invention also relates to mutation specific primers for use in the method and to diagnostic kits containing these primers.

Description

Colorectal cancer is the second most common cause of cancer mortality in the US and is a significant cause of morbidity and mortality world-wide. The American Cancer Society estimated that in 1995 more than 130,000 new cases of colorectal cancer (CRC) would be diagnosed in the US and that there would be 55,300 deaths from the disease. The incidence is higher in men than in women (60.4 versus 40.9 per 100,000 per year). Age-specific incidence and mortality rates show that most cases are diagnosed after 50 years of age. Most patients (65%) present with advanced disease with about 60% of patients having regional or distant metastases at the time of diagnosis. The estimated 5-year survival is 91% in persons with localised disease, 60% in persons with regional spread, and only 6% in those with distant metastases. An individual's lifetime risk of dying of colorectal cancer in the US has been estimated to be 2.6%. CRC onset and its progression have been studied extensively at the molecular and genetic levels and there is a commonly accepted model relating tumour status, classified according to Duke's stage, to specific changes in DNA.

It has recently been concluded that reliable CRC screening procedures require development and that additional research to identify mutated genes in clinical samples is needed. The United States Preventive Services Task Force also recommends screening for CRC for all persons aged 50 and older with annual faecal occult blood testing (FOBT) or sigmoidoscopy or both. The reported sensitivity and specificity of FOBT for detecting colorectal cancer in asymptomatic persons are 26-92% and 90-99%, respectively (usually based on two samples from three different stool specimens). However, the ingestion of foods containing peroxidases, and gastric irritants such as salicylates and other antiinflammatory agents, can produce false-positive test results for neoplasia. Nonneoplastic conditions, such as

haemorrhoids, diverticulosis, and peptic ulcers, can also cause gastrointestinal bleeding that would give rise to false-positive test results. FOBT can also miss small adenomas and colorectal malignancies that bleed intermittently or not at all. Other causes of false-negative results include heterogeneous distribution of blood in faeces, ascorbic acid and other
5 antioxidants that interfere with test reagents, and extended delay before testing stool samples. As a result, when FOBT is performed on asymptomatic persons, the majority of positive reactions are falsely positive for neoplasia. The reported positive predictive value among asymptomatic persons over age 50 is only about 2-11% for carcinoma and 20-30% for adenomas. Assuming a false-positive rate of 1-4%, a person who receives annual FOBT from
10 age 50 to age 75 has an estimated 45% probability of receiving a false-positive result. This large proportion of false-positive results is an important concern because of the discomfort, cost, and occasional complications associated with follow-up diagnostic tests, such as barium enema and colonoscopy. Other tests have been proposed to improve the accuracy of screening for faecal occult blood. Current evidence is equivocal as to whether "HemoQuant"®
15 (SmithKline Diagnostics, Sunnyvale, CA), a quantitative measurement of haemoglobin in the stool, has better sensitivity or specificity than does qualitative FOBT.

Sigmoidoscopic screening in asymptomatic persons detects 1-4 cancers per 1,000 examinations. However, the sensitivity and diagnostic yield of sigmoidoscopy screening varies with the type of instrument. Since only 30% of colorectal cancers occur in the distal 20
20 cm of bowel, and less than half occur in or distal to the sigmoid colon, the length of the sigmoidoscope has a direct effect on case detection. The rigid sigmoidoscope, which has an average depth of insertion of about 20 cm and allows examination to just above the rectosigmoid junction, can detect only about 25-30% of colorectal cancers. The 35 cm flexible sigmoidoscope, however, can visualise about 50-75% of the sigmoid colon. The
25 longer 60 cm instruments have an average depth of insertion of 40-50 cm, reaching the proximal end of the sigmoid colon in 80% of examinations with the capability of detecting 40-65% of colorectal cancers.

Other potential screening tests for colorectal cancer include colonoscopy and barium enema, which appear to have comparable accuracy. About 95% of colorectal cancers
30 are within reach of the colonoscope, and the examination has an estimated 75-95% sensitivity in detecting lesions within its reach. The introduction of flexible fibre-optic instruments has

made sigmoidoscopy more acceptable to patients, however, the procedure is expensive and remains uncomfortable and embarrassing, and therefore many patients may be reluctant to agree to this test. Colonoscopy, which requires sedation and often involves the use of a hospital suite, is more expensive than other screening tests and has a higher risk of anaesthetic and procedural complications.

There are tumour markers that are detected by immunodiagnostic techniques. For example, carcinoembryonic antigen (CEA), an oncofetal glycoprotein antigen with unknown function. Elevated serum levels of CEA correlate with Duke's stage of CRC. Elevated CEA levels are also evident in breast, liver, lung, pancreatic and gastric neoplasms. The limitations of tests based on antibodies raised to such tumour-associated antigens are related to both specificity and sensitivity.

Although there are many examples of nucleic acid changes having potential as tumour markers, their value as clinical tools in cancer diagnosis, staging or even screening, needs to be demonstrated and two important criteria must be met. Firstly, nucleic acids of adequate yield and quality must be extracted from the clinical material; secondly, robust and accurate methods of analysis are required. For reliable tumour genotyping to be useful in disease staging any test has to be adequately validated and there should be demonstrable benefits over current methods.

A number of studies have examined the association of CRC with mutations in the oncogene, *K-ras*. However, there have been significant differences in the reported frequencies of *K-ras* mutations in CRC. Inconsistencies between studies could be due to one or more of several factors. These include the number of tumours investigated, the methods used and the number of individual point mutations tested for. It is therefore difficult to state the true number of CRCs which contain *K-ras* mutations. Some associations between *K-ras* genotype and phenotype have been proposed. For instance, *K-ras* genotypes vary by amino acid substitution and have been categorised into different types. In a recent study, *K-ras* mutations have been shown to be associated with increased risk of relapse and death, with some mutations, such as the codon 12 glycine to valine change being more aggressive than others (Jervoise, H. et al., J.Nat.Cancer Inst., 90 675-684,1998).

In the present invention we have now devised novel diagnostic methods for the detection of *K-ras* mutations based on ARMS. Validated tests for seven *K-ras* point

mutations have been developed and the tests have been applied in a thorough investigation of the incidence of the mutations in tumours from a large assembly of CRC patients.

According to a first aspect of the invention we now provide a diagnostic method for the detection of *K-ras* mutations in cancer, which method comprises contacting a test sample of nucleic acid with a diagnostic primer for a *K-ras* mutation in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when a *K-ras* mutation is present in the sample; and detecting the presence or absence of a *K-ras* mutation by reference to the presence or absence of a diagnostic primer extension product.

Each of the diagnostic primers detects one of the following *K-ras* mutations:

Table 1:

PRIMER NUMBER	MUTATION DETECTED
1	<i>K-ras</i> codon 12 GGT→AGT (glycine → serine)
2	<i>K-ras</i> codon 12 GGT→CGT (glycine → arginine)
3	<i>K-ras</i> codon 12 GGT→TGT (glycine → cysteine)
4	<i>K-ras</i> codon 12 GGT→GAT (glycine → aspartic acid)
5	<i>K-ras</i> codon 12 GGT→GCT (glycine → alanine)
6	<i>K-ras</i> codon 12 GGT→GTT (glycine → valine)
7	<i>K-ras</i> codon 13 GGC→GAC (glycine → aspartic acid)

It is believed that the diagnostic methods of the invention may be used to detect a range of cancers, for example non-small cell lung cancer, pancreatic cancer and, in particular, colorectal cancer.

The test sample of nucleic acid is conveniently a sample of blood, faeces, sputum, colonic lavage, bronchial lavage or other body fluid, or tissue obtained from an individual. The individual is conveniently human, preferably *Homo sapiens*. It will be appreciated that

the test sample may equally be a nucleic acid sequence corresponding to the sequence in the test sample. That is to say that all or a part of the region in the sample nucleic acid may firstly be amplified using any convenient technique such as PCR before use in the method of the invention.

- 5 Any convenient enzyme for polymerisation may be used provided that it does not affect the ability of the DNA polymerase to discriminate between normal and mutant template sequences to any significant extent. Examples of convenient enzymes include thermostable enzymes which have no significant 3'-5' exonuclease activity, for example *Taq* DNA polymerase, particularly "Ampli Taq Gold"TM DNA polymerase (PE Applied Biosystems),
10 Stoffel fragment, or other appropriately N-terminal deleted modifications of *Taq* or *Tth* (*Thermus thermophilus*) DNA polymerases.

- We have now devised primers for the above *K-ras* point mutations which have been shown to detect the specific mutations reliably and robustly. Therefore in a further aspect of the invention we provide diagnostic primers comprising the sequences given below and
15 derivatives thereof wherein 6-8 of the nucleotides at the 3' end are identical to the sequences given below and wherein up to 10, such as up to 8, 6, 4, 2, 1, of the remaining nucleotides may be varied without significantly affecting the properties of the diagnostic primer. Conveniently, the sequence of the diagnostic primer is exactly as written below.

20 **Table 2:**

PRIMER NUMBER	MUTATION DETECTED	PRIMER SEQUENCE
1	K12 Serine	CTGAATATAAACTTGTGGTAGTTGGAGCCA
2	K12 Arginine	CTGAATATAAACTTGTGGTAGTTGGAGCCC
3	K12 Cysteine	CTGAATATAAACTTGTGGTAGTTGGAGCAT
4	K12 Aspartic acid	TATCGTCAAGGCACTCTTGCCTACGCCTT
5	K12 Alanine	TATCGTCAAGGCACTCTTGCCTACGCCTG
6	K12 Valine	TATCGTCAAGGCACTCTTGCCTACGCCTA
7	K13 Aspartic acid	CGTGTATCGTCAAGGCACTCTTGCCTACCT

The primers may be manufactured using any convenient method of synthesis. Examples of such methods may be found in standard textbooks, for example "*Protocols For Oligonucleotides And Analogues: Synthesis And Properties*," Methods In Molecular Biology Series; Volume 20; Ed. Sudhir Agrawal, Humana ISBN: 0-89603-247-7; 1993; 1st Edition.

It will be appreciated that any of the above diagnostic methods may, if appropriate, also be configured so that extension of the diagnostic primer indicates the absence of the K-ras mutation.

In many situations, it will be convenient to use a diagnostic primer of the invention with a further amplification primer in one or more cycles of PCR amplification. A convenient example of this aspect is set out in our European patent number EP-B1-0332435. The further amplification primer is either a forward or a reverse common primer. The forward primer: GTACTGGTGGAGTATTTGATAGTGTATTAACC

is conveniently used in combination with one or more of diagnostic primers 4, 5, 6 & 7 and the reverse primer:

CTCATGAAAATGGTCAGAGAAACCTTTATC

is conveniently used in combination with one or more of diagnostic primers 1, 2, & 3.

Any convenient control primer may be used. We have selected control primers from two unrelated regions of the genome, namely part of exon 4 of the cystic fibrosis transmembrane regulator gene, and part of exon II and intron III of the human α_1 -antitrypsin gene.

It will be appreciated that further useful diagnostic tests will be provided by combining 2 or more diagnostic primers for the 7 loci indicated above. Any appropriate combination of the above primers may be used. Particular combinations include primers 1+2, primers 1+2+3, primers 4+5, primers 4+5+6, and primers 4+5+6+7. Each of these combinations will also include the addition of the appropriate common primer.

The diagnostic methods of the invention as outlined above may be conveniently effected in one or more reaction vessels. Where more than one diagnostic mutation is to be assayed the diagnostic primer (and corresponding amplification primer) are provided in individual tubes i.e. one tube per mutation. Alternatively, the reactions may be multiplexed,

that is to say that all the diagnostic primers and amplification primers are in one tube (see EP-B1-0332435).

A variety of methods may be used to detect the presence or absence of diagnostic primer extension products and/or amplification products. These will be apparent to the person skilled in the art of nucleic acid detection procedures. Preferred methods avoid the need for radiolabelled reagents. Particular detection methods include "Taqman"TM product detection, for example as described in patent numbers US-A-5487972 & US-A-5210015; "Molecular Beacons"[®] product detection, outlined in patent number WO-95/13399 and surface enhanced raman resonance spectroscopy (SERRS), outlined in our patent application WO 97/05280. Further preferred detection methods include ARMS linear extension (ALEX) and PCR with ALEX as described in Example 4 of this application. Conveniently, real-time detection is employed.

One or more of the diagnostic primers of the invention may be conveniently packaged with instructions for use in the method of the invention and appropriate packaging and sold as a kit. The kits will conveniently include one or more of the following: appropriate nucleotide triphosphates, for example dATP, dCTP, dGTP, dTTP, a suitable polymerase as previously described, and a buffer solution.

The invention will now be illustrated but not limited by reference to the following Example, Tables and Figures.

Figure Legends

Legend to Figure 1.

The *K-ras* genomic DNA sequence; wild-type codons 12 and 13 are shown in bold type. Filled arrows (➔ and ➜) identify the 3' end of the ARMS primers designed to amplify individual *K-ras* mutated sequences. Open arrows (⇨ and ⇩) identify the 3' ends of the flanking intron. The ARMS primers were designed to detect point mutations of *K-ras* codon 12 glycine (GGT) to arginine (CGT), cysteine (TGT), serine (AGT), valine (GTT), aspartic acid (GAT) and alanine (GCT) and point mutation of codon 13 glycine (GGC) to aspartic acid (GAC). The direction of the arrow heads indicates sense (➔ and ⇨) and antisense (➜ and ⇩) primers. ARMS reaction products derived from the intron-specific primer cpA (⇨) and any of the antisense ARMS primers (➜) are 158 base-pairs (codon 12 mutations) or 161 base-pairs

(codon 13 mutation). ARMS amplicons derived from the intron-specific primer cpB (⇐) and any of the sense ARMS primers (⇒) are 190 base-pairs.

Legend to Figure 2

Development of the K12 Asp (panel A), K12 Cys (panel B), K13 Asp (panel C) and K12 Val (panel D) ARMS tests. In each case lanes 1 and 2 are tests on tumour DNA; lane 3, 10^3 genome equivalents wild-type DNA; lane 4, 10^3 genome equivalents mutant (cell line) DNA; lane 5, 10^3 genome equivalents wild-type DNA plus 10^2 genome equivalents mutant DNA; lane 6, no DNA.

Legend to Figure 3

Example of a typical ARMS analysis on tumour DNA. DNA from selected Dukes' C tumours was tested using the K12 Ala ARMS test. Lane 1, Φ x174/*Hae*III size markers; lanes 2-14, tumours 1305, 5, 6, 13, 20, 21, 23, 39, 121, 122, 135, 137 and 142 respectively; lane 15, 10^5 genome equivalents SW116 cell line DNA; lane 16, no DNA.

Legend to Figure 4

Three-primer PCR with cloned *K-ras* exon I amplicons. Lanes 1 and 48, Φ x174/*Hae*III size markers; lanes 2-46, PCR aliquots from reactions carried out with the M13 forward and reverse primers and the ARMS primer that gave the preliminary ARMS result. High molecular weight bands signify the presence of cloned insert; low molecular weight bands signify that no insert is present in the clone. Intermediate molecular weight bands are derived from the respective ARMS primer and one or other of the M13 primers, the slightly different sizes of these bands signify the use of either a sense or an antisense ARMS primer. Lane 47, no DNA.

Legend to Figure 5

Control data graphs showing homogeneous detection of *K-ras* mutant sequences using ARMS coupled with amplicon detection by Molecular Beacons. Data points were subjected to regression analysis with 98% confidence limits. Data for each *K-ras* mutation is shown as follows:

Figure 5a K-*ras* codon 13 Gly to Asp

Figure 5b K-*ras* codon 12 Gly to Asp

Figure 5c K-*ras* codon 12 Gly to Val

Figure 5d K-*ras* codon 12 Gly to Ala

5 Figure 5e K-*ras* codon 12 Gly to Ser

Figure 5f K-*ras* codon 12 Gly to Arg

Figure 5g K-*ras* codon 12 Gly to Cys

Example 1

10 In Example 1 we report a study of the incidence of K-*ras* mutations in colorectal cancer using 301 DNA samples extracted from a colorectal tumour bank. In this study mutations within codons 12 and 13 of the K-*ras* oncogene were investigated using ARMS (Newton C.R. et al., Nucl. Acids Res., 17: 2503-2516, 1989). Direct DNA sequencing (Newton C.R. et al., Nucl. Acids Res., 16: 8233-8243, 1988), and sequencing of cloned amplicons were
15 then performed to assess the ARMS test results. In nearly half of the cases the direct sequencing result detected K-*ras* wild-type sequence only, this was in contrast to the ARMS findings where mutations in codons 12 and 13 were detected. When K-*ras* exon I amplicons were cloned into *E.coli*, the ARMS result was consistently in accord with the sequence of the cloned material with the exception of one tumour which harbored a mutation of five
20 consecutive nucleotides that was detected by three of the ARMS primers. Our data show that ARMS is a sensitive test for detecting under-represented nucleic acid sequences. We also demonstrate that the technique is ideally suited to the detection of tumour DNA markers supplying genotype information specific to pre-diagnosed tumours.

25 Materials And Methods

(1) DNA Extraction From Tumour Bank Samples.

DNA was extracted from 301 frozen tissue samples. Positive selection of samples comprised the exclusion of adenomas and tumours from familial adenomatous polyposis patients. Altogether, the samples comprised colorectal lesions excised between January 1985 and
30 January 1995, 6 of which were adenomas. Of the remaining cancers 31 were classified as

Dukes' stage A, 135 as Dukes' B and 129 as Dukes' C (Dukes C.E., J. Pathol. Bacteriol., 35: 323-332, 1932).

Each frozen specimen was sectioned by cryostat, 5µm was taken for haematoxylin and eosin staining and 3 or 4 parallel 10µm sections were transferred to sterile tubes and
5 stored at -70°C. Fresh blades were used for each sample.

DNA extraction comprised thawing sections on ice followed by the addition of sufficient sterile lysis buffer [10mM Tris-HCl (pH 7.5), 20% sodium dodecyl sulfate, 50µg/ml proteinase K] to saturate the material. After an overnight digestion at 37°C a standard phenol/chloroform
10 purification and ethanol precipitation was carried out (Sambrook J., Fritsch E.F. & Maniatis T. (eds) *Molecular Cloning: A Laboratory Manual*. 2nd edition Cold Spring Harbor Laboratory Press (1989)). The resulting DNA was resuspended in 200µl 10mM Tris-HCl (pH 7.5). DNA samples were then stored at -70°C prior to quantification and K-ras mutation analysis.

15 (2) K-ras Mutation ARMS Tests.

Individual ARMS tests were developed to detect specific point mutations in the K-ras oncogene. The 3'-terminal base of each of seven ARMS oligonucleotide primers was complementary to one of the common mutations of codons 12 or 13 of the K-ras oncogene occurring in CRCs (Breivik J. et al., Br. J., Cancer, 69: 367-371, 1994), (Fig. 1). In addition to
20 the ARMS primers, a common primer complementary to K-ras intron sequence was included (Fig. 1). ARMS and common primer sequences are shown in Table 3.

Two other primer pairs were also present in each test to give amplification control products. Their sequences were:

5'-TATATGTGCCATGGGGCCTGTGCAAGGAAG-3' and
25 5'-CTCCTACACCCAGCCATTTTGGC-3' which amplify part of exon four of the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Riordan J.R. et al., Science, 245:1066-1073, 1989) and
5'-GGGCCTCAGTCCCAACATGGCTAAGAGGTG-3' and
5'-CCCACCTTCCCCTCTCTCCAGGCAAATGGG-3' which amplify a part of each of exon
30 II and intron III of the human α_1 -antitrypsin gene (Newton C.R. et al., Nucl. Acids Res., 16: 8233-8243, 1988).

Normal human DNA was extracted from the blood of healthy volunteers (Ferrie R.M. et al., Am. J. Hum. Genet., 51: 251-262, 1992). *K-ras* mutated DNA samples were extracted from tumour derived cell lines as shown in Table 4. The *K-ras* mutation for each cell line DNA was confirmed by direct DNA sequencing as described below for tumour derived
5 DNA samples. The cell line DNAs were then used to define amplification conditions that conferred specificity to each ARMS reaction when the following criteria were applied.

First, after the DNA amplification reaction and agarose gel electrophoresis in the presence of 0.5µg/ml ethidium bromide (Sambrook J., Fritsch E.F. & Maniatis T., *op.cit.*), the test should give a visible ARMS band only when either 10^2 genome equivalents of the
10 appropriate mutant DNA with 10^5 equivalents of normal DNA is combined or when 10^2 genome equivalents of mutant DNA alone is tested. A genome equivalent meaning here the amount of genomic DNA per cell.

In addition, there should be no visible ARMS product from any primer when 10^5 genome equivalents of normal DNA is tested in isolation.

15 The annealing temperature and number of cycles used for each ARMS reaction is shown in Table 5. All amplification reactions were performed applying the commonly accepted precautions for avoiding carry-over contamination (Kwok S. & Higuchi R., Nature, 339: 237-238, 1989)

The amount of DNA extracted from each tumour sample was measured by
20 fluorescence following intercalation of the Hoechst dye 33258 (Riley J. et al., Nucl. Acids Res., 17: 8383, 1989).

ARMS reactions containing these DNAs (1µl each) were performed in 50µl buffer comprising 10mM Tris-HCl, (pH 8.3), 1.2mM MgCl₂, 50mM KCl, 0.01% gelatin and dNTPs (100mM each). The reactions also contained mutation-specific and the appropriate intron-
25 specific primers (1µM each) as shown in Fig 1. The CFTR gene amplimers were 0.075µM each and the α_1 -antitrypsin primers were 0.025µM each.

Hot-start PCR (D' Aquila R.T. et al., Nucl. Acids Res., 19: 3749, 1991; Chou Q. et al., Nucl. Acids Res., 20: 1717-1723, 1992) was performed throughout by adding a layer of white mineral oil and heating the samples at 94°C for 5 minutes before adding *Taq* DNA
30 polymerase (1 unit). Thermal cycling comprised of 35 or 36 cycles (Table 5) of 94°C, 1 minute

denaturation; 58°-63°C, 1 minute annealing (Table 5); 72°C, 1 minute extension. This was followed by a final incubation at 72°C for 10 minutes.

Any sample which failed to amplify, identified by the absence of control bands, was re-tested until data for all tumour samples with all 7 tests was complete.

5

(3) K-ras ARMS Test Validation: Direct Sequencing Of Tumour Derived DNA.

Tumour derived DNA (2µl) was amplified in 50µl reactions which comprised 10mM Tris-HCl (pH 8.3), 100mM tetramethylammonium chloride, 3mM MgCl₂, 0.05% Tween-20, 0.05% Nonidet NP40, dNTPs (200µM each) and 2.5 units of *Taq* DNA polymerase.

10 Each reaction also contained the forward primer

5'-CTGGATCTAGACTCATGAAAATGGTCAGAGAAACCTTTATC-3' and the reverse primer

5'-CCTCGGAATTCGTACTGGTGGAGTATTTGATAGTGTATTAACC-3' (500nM each) which generate an amplicon from exon I of the K-ras oncogene with flanking *Xba*I and *Eco*RI

15 restriction enzyme recognition sites.

Reactions were overlaid with mineral oil (50 µl) and amplified over 35 cycles of 94°C, 60°C, 72°C (1 minute each). After electrophoresis through a 2% metaphor agarose gel (FMC Bioproducts), the exon I bands were excised and purified using a "Wizard"® DNA purification kit (Promega). Typical yields were 1-5µg in 50µl.

20 The purified products were sequenced by direct incorporation of [α^{35} S]dATP (Amersham) using a modified version (Green P.M. et al., The EMBO Journal, 8: 1067-1072, 1989) of the "Sequenase 2.0"® DNA sequencing kit (Amersham). Annealing mixtures also contained template DNA (6µl), sequencing primer (1µl, 500ng) and dimethylsulphoxide (1µl), (Sigma). Each labeling reaction was supplemented with 0.2units DNA polymerase I, Klenow
25 fragment (labeling grade, Boehringer Mannheim) (Redston M.S. & Kern S.E., Biotechniques, 17: 286-288, 1994). Sequencing reactions were run on 6% polyacrylamide gels which were subsequently dried and autoradiographed.

(4) K-ras ARMS Test Validation: Cloning and Sequencing of Tumour DNAs.

30 An aliquot of each amplicon prepared for direct DNA sequencing was also ligated into the vector pGEM-T (Promega) at 17°C overnight. A 2µl aliquot from each ligation

mixture was used to transform competent *E.coli* JM109 cells (Promega). These were plated and blue/white screened according to the supplier's instructions. White colonies were transferred to 10 µl of sterile distilled water.

PCR using the conditions described above, but for 25 cycles, was carried out to test simultaneously for the presence of an insert and the *K-ras* mutation status of any insert. The amplimers in each reaction were the M13 5'-GTTTTCCCAGTCACGAC-3' (forward), 5'-CAGGAAACAGCTATGAC-3' (reverse) primers and the ARMS primer that initially identified the mutation. Amplification products were then visualised on a 3% agarose gel.

Clones with inserts detected by the PCR screen were transferred to 1ml of LB broth and grown overnight at 37°C. An aliquot (100µl) from each was inoculated into a further 1ml broth and grown for 3-4 h at 37°C. Ten pfu/cell of M13KO7 helper phage were then added to each culture. After 1h at room temperature LB broth (9ml) containing 70µg/ml kanamycin and 100µg/ml ampicillin was added and the culture incubated overnight at 37°C. Virus particles were isolated by polyethylene glycol 6000/NaCl precipitation and single-stranded DNA was isolated by phenol chloroform extraction followed by ethanol precipitation (Sambrook J., Fritsch E.F. & Maniatis T., *op. cit.*). DNA sequencing was performed using the M13 forward primer as described above.

(5) Statistical Methods.

The influence of sex of patient and Dukes' stage on the proportion of *K-ras* mutations for all 301 tumours was analysed by logistic models (McCullagh P. & Nelder J.A., Generalised linear models. New York: Chapman and Hall. 1983). The prevalence of these factors in the tumour bank samples was analysed by log-linear models. For this analysis the adenomas were excluded because of the deliberate selection against these samples.

Results

(1) Histological Analyses.

Histological examination of the haematoxylin and eosin stained material confirmed the presence of tumour cells in at least 90% of the sample in each case (data not shown).

(2) ARMS Test Development.

Thermal cycling and primer annealing conditions were determined empirically having imposed the specificity criteria described above, these are shown in Table 5. An example showing the specificity of each of four of the tests is shown in Figure 2.

5

(3) DNA Yield From Tumour Extracts.

The maximum and minimum DNA yields were 124.5µg (622.5 ng/µl) and 1.5µg (7.5 ng/µl) respectively, approximating to between 1.25×10^5 and 1.5×10^3 human diploid genome equivalents/µl.

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Six of the Dukes' C samples failed to give sufficient DNA to reach the threshold of detection but this did not preclude ARMS analyses of these samples. In general, a lower yield of DNA was associated with the extracts from Dukes' C classified tumours. The mean DNA yield was 25.6µg (128.0 ng/µl), approximately 2.5×10^4 genome equivalents/µl after discounting the six samples that failed to give measurable quantities of DNA extract.

15

(4) ARMS Tests And Direct Sequencing.

A typical ARMS result is shown in Figure 3. A summary of the *K-ras* mutations detected using ARMS, the Dukes' stage of the tumour and the direct DNA sequencing result for 30 of the tumour DNAs examined is shown in Table 6.

20

(5) Quantitative Analysis of *K-ras* Mutations.

Three primer PCR results used to classify clones from a selection of tumour DNAs are shown in Figure 4. Table 7 provides an analysis of the ARMS data, direct DNA sequencing and clone analyses from equivalent samples.

25

(6) *K-ras* Mutational Analysis of the 301 Tumour Bank Samples.

Since the majority of CRCs are classified as either Dukes' B or C at the time of surgery a relatively smaller number of Dukes' A (31 in total, 10.3%) cancers was analysed. Adenomas comprised 6 in total (2%) for the same reason and also because of the deliberate selection against these tumours. For the 295 non-adenoma tumours, there were more from male than female patients (183 male, 118 female), 36% had *K-ras* mutations and there were

30

relatively higher numbers of severe disease patients 31, 135 and 129 Dukes' stages A, B and C respectively. There were relatively more severe male than female patients (male:female ratio for each Dukes' stage is; A, 15:16; B, 74:61; C, 90:39; $p < 0.05$). For the proportion of *K-ras* mutations, there was evidence that this was less ($p < 0.05$) for males (31.7%) than females (43.2%). There was no evidence that the proportions differed across Dukes' stages either for the sexes separately or combined (combined proportions, Dukes' A, 13:18, 42%; Dukes' B, 47:88, 35%; and Dukes' C 48:81, 37%; $p > 0.05$). Table 6 shows the results on which the *K-ras* codons 12 and 13 mutational analysis was made and the relative *K-ras* mutation frequencies.

The aims of our study were to develop validated tests for seven *K-ras* point mutations and to apply them in a thorough investigation of the incidence of the mutations in tumours from a large assembly of CRC patients. As part of the ARMS test validation process we employed the sequencing strategy described in Materials and Methods. Our initial approach to directly sequence PCR amplicons verified the ARMS result in approximately half of the tumour DNA samples investigated. One possible explanation for this could be that the ARMS tests failed to discriminate mutated from normal sequences. However, the tumours were not microdissected, but a large proportion of tumour cells relative to normal tissue was identified by histology. Assuming that the tumour cells were monoclonal for any given *K-ras* mutation the ratio of mutant to normal DNA might then be expected to be relatively high. *K-ras* is an oncogene, thus there is no reason to suppose that the normal copy of the gene should not be present in *K-ras* mutant tumour cells, unlike the occurrence of allele loss with tumour suppressor genes. Therefore, when taking into account the presence of normal DNA, the mutant sequences could actually be expected to account for only a small proportion of the total DNA of the sample and so go undetected by direct DNA sequencing. This is upheld by our observations and we therefore concluded that direct DNA sequencing was inappropriate for substantiating the ARMS results. This was confirmed by the second stage of validation where cloned amplicons of *K-ras* exon I were sequenced. Here, the DNA sequence data gave comparable results to those derived by ARMS. The results from this stage of validation therefore indicate the use of a method that will detect mutations that are under-represented against a background of wild-type alleles. The ARMS tests described herein were validated to a sensitivity of at least 1 mutant *ras* sequence in 10^3 wild-type sequences. In fact, control

reactions for tumour DNA analysis had routinely lower ratios of mutant to wild-type input DNA. Other groups have reported tests based on the same principles as the ARMS tests described here. Detection levels of mutant *ras* sequences present at as low as 1 in 10⁵ wild-type sequences have been reported. This ability of the technique to detect rare mutations in a background of normal DNA demonstrates its potential role in screening or in the monitoring of residual disease.

ARMS is a simple and accurate method and has several benefits over other PCR-based mutation detection systems. Specifically, the technique does not require the use of radioisotopes or the multiple probing of immobilised PCR amplicons or cloned PCR amplicons. ARMS avoids the need for DNA sequencing of single-strand conformation polymorphism products, a procedure that could be expected to be constrained by sequence under-representation as discussed above. Similarly, under-represented mutant sequences could go undetected using PCR in conjunction with restriction fragment length polymorphism which is limited to low cycle numbers for the PCR to avoid false positive results. Studies that have examined the elimination of the *Bst*NI restriction site at codon 12 in some *K-ras* mutations are severely limited because these tests rely on a *Bst*NI restriction digest part way through PCR cycling. Previously generated amplicons therefore have the potential to cause carry-over contamination when PCR is resumed. ARMS can be performed under conditions in which carry-over contamination is avoided, as in the present study, allowing the use of high PCR cycle numbers and resulting in exceptionally high detection sensitivity.

One DNA sample analysed gave a positive result in more than one ARMS test. Sample 1342 was derived from a Dukes' B rectal tumour from a 49 year old male. This sample gave a positive result with the K12 glycine (GGT) to arginine (CGT), serine (AGT) and alanine (GCT) tests. Direct sequence analysis was non-informative but DNA sequencing of each of the cloned PCR amplicons revealed a five nucleotide mutation. The normal sequence for codons 11 to 13 (GCT GGT GGC) was changed to GCC ACC AGC such that there is a reversed inversion of the last nucleotide of codon 11 to the first nucleotide of codon 13 resulting in a K12 glycine to threonine and K13 glycine to serine mutant protein. Such an occurrence would possibly be the result of an aberrant recombinogenic event. The DNA sequencing results therefore exclude the possibility that tumour 1342 is polyclonal for more than one *K-ras* point mutation. None of the tumours analysed were found to have more than one *K-ras* mutation,

also the overall frequency of *K-ras* mutation does not increase significantly between Dukes' stages. This supports the model that *K-ras* mutation is a relatively early event in the progression of CRC through Dukes' stages A to C. Because only six adenomas were included in this study the exact timing of *K-ras* mutation in the adenoma to carcinoma progression was not addressed.

The male to female split of the total non-adenoma patients and the severity of their disease in association with their *K-ras* mutation status was examined. There was no evidence of differing proportions of tumours with or without *K-ras* mutations for the sexes either separately or overall in terms of the disease stage. There were more *K-ras* negative males compared to females, and the males presented with a more severe stage of the disease. However, it should be realised that these observations could be influenced by other factors such as the catchment area from which the patients were drawn. Similarly, the difference in severity of the disease noted between the tumours of males and females could be related to the male/female attitude to the disease and to when they first consulted their primary care clinician.

The value of *K-ras* mutations as a marker of malignancy will depend on several factors, not least being the frequency of tumours of a given type, such as CRC, that carry the mutation. As this study has found the frequency of *K-ras* mutations to be approaching 40%, additional markers for CRC would be required for general screening purposes if all CRCs were to be identified using ARMS.

Tables

Table 3: ARMS primer and common primer DNA sequences.

Primer	Primer sequence (5' - 3')
ARMS K12 Val	TATCGTCAAGGCACTCTTGCCTACGCCTA
ARMS K12 Cys	CTGAATATAAACTTGTGGTAGTTGGAGCAT
ARMS K12 Ser	CTGAATATAAACTTGTGGTAGTTGGAGCCA
ARMS K12 Arg	CTGAATATAAACTTGTGGTAGTTGGAGCCC
ARMS K12 Asp	TATCGTCAAGGCACTCTTGCCTACGCCTT
ARMS K12 Ala	TATCGTCAAGGCACTCTTGCCTACGCCTG

Primer	Primer sequence (5' - 3')
ARMS K13 Asp	CGTGTATCGTCAAGGCACTCTTGCCTACCT
Common primer (cpA)	GTACTGGTGGAGTATTTGATAGTGTATTAACC
Common primer (cpB)	CTCATGAAAATGGTCAGAGAAACCTTTATC

Table 4: Cell lines used in the development and validation of the K-ras ARMS test.

Mutation	Cell line	Source
12Ser	A549	ATCC ref CRL-7909
12Cys	MIA PaCa-2	ATCC ref CRL-1420
12Arg	PSN-1	Yamada H. et al., Biochem. Biophys. Res. Commun., 140: 167-173, 1986
12Ala	SW1116	ATCC ref CCL-233
12Val	Capan 2	ATCC ref HTB-80
12Asp	Panc 1	ATCC ref CRL-1469
13Asp	HCT116	ATCC ref CCL-247

5 Table 5: Annealing temperature and PCR cycle number for each K-ras mutation-specific ARMS primer.

Mutation	Annealing temperature (°C)	Cycles
12Ser	60	36
12Cys	60	35
12Arg	61	35
12Ala	58	35
12Val	60	35
12Asp	63	35
13Asp	63	35

Table 6: The ARMS test, direct sequencing and clone analysis results grouped according to the ARMS primer(s) found to generate K-ras amplicons

Tumour	Dukes' Stage	ARMS result	Direct DNA sequence result
6	C	K12 Arg	K12 Arg
23	C	K12 Cys	wt
72	C	K13 Asp	K13 Asp
178	C	K13 Asp	K13 Asp
188	A	K12 Val	wt
202	B	K12 Asp	wt
214	B	K12 Cys	wt
302	C	K13 Asp	wt
328	B	K12 Asp	K12 Asp
357	B	K12 Asp	K12 Asp
406	B	K12 Val	K12 Val
436	B	K12 Val	wt
561	C	K12 Val	K12 Val
565	B	K12 Asp	wt
596	C	K13 Asp	wt
598	B	K12 Asp	K12 Asp
734	C	K12 Asp	K12 Asp
777	B	K12 Val	wt
863	B	K13 Asp	K13 Asp
980	C	K12 Ser	K12 Ser
982	B	K12 Ser	K12 Ser
1076	C	K12 Asp	K12 Asp
1182	B	K12 Val	K12 Val
1210	A	K12 Val	wt
1253	C	K12 Cys	wt
1257	A	K12 Cys	K12 Cys

Tumour	Dukes' Stage	ARMS result	Direct DNA sequence result
1261	C	K12 Val	K12 Val
1271	C	K12 Ser	K12 Ser
1289	B	K12 Val	wt
1342	B	K12 Ser+ Arg+Ala	Multiple

Table 7: Direct and cloned sequence results from tumour DNAs grouped according to the ARMS primer that initially characterized the K-ras mutation harbored by the tumour.

Tumour DNA	ARMS result	Direct sequence result	Frequency of mutation in clones	Sequence of ARMS positive clones
1271	12 Ser	12 Ser	5 of 27 (19%)	12 Ser
1342	12 Ser	unclear	16 of 28 (57%)	5 mutations
23	12 Cys	wt	14 of 38 (37%)	12 Cys
214	12 Cys	wt	12 of 33 (36%)	12 Cys
500	12 Cys	wt	10 of 33 (30%)	12 Cys
530	12 Cys	wt	2 of 34 (6%)	12 Cys
1253	12 Cys	wt	21 of 43 (49%)	12 Cys
6	12 Arg	12 Arg	4 of 10 (40%)	12 Arg
957	wt	wt	0	no +ve clones
1342	12 Arg	unclear	0	no +ve clones
1342	12 Ala	unclear	not done	not done
188	12 Val	wt	8 of 39 (21%)	12 Val
436	12 Val	wt	11 of 33 (33%)	12 Val
556	12 Val	12 Val	12 of 37 (32%)	12 Val
777	12 Val	12 Val	4 of 38 (10%)	12 Val
1210	12 Val	wt	3 of 42 (7%)	12 Val
1289	12 Val	12 Val	10 of 38 (26%)	12 Val

Tumour DNA	ARMS result	Direct sequence result	Frequency of mutation in clones	Sequence of ARMS positive clones
177	12 Asp	wt	15 of 41 (37%)	12 Asp
202	12 Asp	wt	2 of 12 (21%)	12 Asp
357	12 Asp	12 Asp	20 of 41 (49%)	12 Asp
410	12 Asp	wt	7 of 37 (19%)	12 Asp
546	12 Asp	wt	3 of 39 (8%)	12 Asp
565	12 Asp	wt	2 of 34 (6%)	12 Asp
302	13 Asp	wt	4 of 41 (10%)	13 Asp
596	13 Asp	wt	5 of 24 (21%)	13 Asp

Table 8: Analysis of the frequencies of the K-ras mutations detected using ARMS from all samples from the CRC tumour bank.

Tumour	Frequencies (male patients)		Frequencies (female patients)		Frequencies (all patients)	
	K-ras +ve	K-ras -ve	K-ras +ve	K-ras -ve	K-ras +ve	K-ras -ve
Adenoma	1 of 4 (25.00 %)	3 of 4 (75.00 %)	0 of 2 (0.00 %)	2 of 2 (100.00 %)	1 of 6 (16.66 %)	5 of 6 (83.33 %)
Dukes' A	5 of 15 (33.33 %)	10 of 15 (66.66 %)	8 of 16 (50.00 %)	8 of 16 (50.00 %)	13 of 31 (41.93 %)	18 of 31 (58.06 %)
Dukes' B	24 of 74 (32.43 %)	50 of 74 (67.57 %)	23 of 61 (37.70 %)	38 of 61 (62.30 %)	47 of 135 (34.81 %)	88 of 135 (65.19 %)
Dukes' C	28 of 90 (31.11 %)	62 of 90 (68.89 %)	20 of 39 (51.28 %)	19 of 39 (48.72 %)	48 of 129 (37.21 %)	81 of 129 (62.79 %)

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Table 9: K13/K12 RAS Test.

Reagent mixes for each test are prepared and divided into aliquots (40 µl) at 1.25 times final concentration.

Reagent	Final concentration
Upper control AAT primers	0.03125 μ M
Lower control CF exon 4 primers	0.09375 μ M
ARMS primer	1.25 μ M
Common primer	1.25 μ M
Reagent	Final concentration
dNTPs	125 μ M
ARMS buffer	1.25X ^a

^a1X ARMS buffer = 10mM Tris-HCl, (pH 8.3) 1.2mM MgCl₂, 50mM KCl, 0.01% gelatin

5 Table 10: K13/K12 RAS Test.

Reaction mix aliquots are transferred to reaction tubes according to individual test:

Mutation detected	Tube type
K12 VALINE	Natural thin wall tubes
K12 CYSTEINE	Pink thin wall tubes
K12 SERINE	Green thin wall tubes
K12 ARGININE	Blue thin wall tubes
K12 ASPARTATE	Yellow thin wall tubes
K12 ALANINE	Purple thin wall tubes
K13 ASPARTATE	Orange thin wall tubes

10 Table 11: Control primer sequences

The lower control primers amplify part of exon four of the human cystic fibrosis transmembrane conductance regulator (CFTR) gene and the upper control primers amplify a part of each of exon II and intron III of the human α_1 -antitrypsin gene.

Primer function	Primer sequence (5' - 3')
Upper control 1	GGGCCTCAGTCCCAACATGGCTAAGAGGTG
Upper control 2	CCCACCTTCCCCTCTCTCCAGGCAAATGGG
Lower control 1	TATATGTGCCATGGGGCCTGTGCAAGGAAG
Lower control 2	CTCCTACACCCAGCCATTTTTTGGC

Table 12: K13/K12 RAS test using "AmpliTaq Gold"™ DNA polymerase

- 5 To initiate each test AmpliTaq Gold™ (P. E. Applied Biosystems) is diluted to 5µl in 1X ARMS buffer and the test DNA (5µl) is added.

Mutation detected	AmpliTaq Gold® (units/5µl)
K12 VALINE	1.0
K12 CYSTEINE	1.0
K12 SERINE	0.5
K12 ARGININE	1.0
K12 ASPARTATE	0.6
K12 ALANINE	1.0
K13 ASPARTATE	0.6

Table 13: Final concentrations of the test reagents

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Thermal cycling conditions for all tests are: 94°C, 20 min; followed by 36 cycles of 94°C, 45 sec; 63°C, 45 sec; 72°C, 45 sec followed by a final incubation at 72°C, 10 min.

- 15 Diagnostic and control amplicons are visualised after electrophoresis of reaction mix aliquots (20µl) through 3% Nusieve (FMC BioProducts) agarose gels against ΦX174/*Hae*III digested DNA size marker.

Reagent	Final concentration
Upper control AAT primers	0.025 μ M
Lower control CF exon 4 primers	0.075 μ M
ARMS primer	1.0 μ M
Common primer	1.0 μ M
Reagent	Final concentration
dNTPs	100 μ M
ARMS buffer	1.125X

Example 2

Identification of k12 *ras* mutations by arms linear extension ("ALEX"TM)

5

Steps 1-15

Step 1: K-*ras* exon I is amplified by PCR in a final volume of 100 μ l. Two reactions are carried out for each sample, in each reaction one of the primers is biotinylated. This produces PCR products in which either the sense or anti-sense strand is biotinylated.

10 **Table 14: Final concentrations of added reagents**

Reagent	Final concentration
Forward primer	1.00 μ M
Reverse primer	1.00 μ M
dNTPs	100 μ M
ARMS buffer	1.125X

Table 15: Primer sequences

Primer	Primer sequence (5' - 3')
Forward primer (Biotinylated)	GTACTGGTGGAGTATTTGATAGTGTATTAACC

Primer	Primer sequence (5' - 3')
Forward primer (Unmodified)	As above
Reverse primer (Biotinylated)	CCTCTATTGTTGGATCATATTCGTCCA
Reverse primer (Unmodified)	ACTCATGAAAATGGTCAGAGAAACCTTTATC

Step 2: Test DNA (1µl) is added.

Step 3: To initiate each test “AmpliTaq”™ DNA polymerase (P. E. Applied Biosystems) diluted to 2 units in 10µl in 2X ARMS buffer is added.

5

Step 4: Thermal cycling conditions for all tests are: 94°C, 5 min; prior to “Amplitaq”™ DNA polymerase addition then 50 cycles of 94°C, 1 min; 60°C, 1 min; 72°C, 1 min followed by a final incubation at 72°C, 10 min.

10 **Step 5:** Diagnostic amplicons are visualised after electrophoresis of reaction mix aliquots (5µl) through 2% Nusieve 3:1 agarose (FMC BioProducts) agarose gels against ΦX174/*Hae*III digested DNA size marker.

15 **Step 6:** The amplicon from the remainder of each reaction mixture is purified, for example, using QiaQuick PCR purification kit (Qiagen) according to the manufacturer’s protocol.

20 **Step 7:** PCR products (about 10¹² molecules) are immobilised in streptavidin-coated microwell plates (Kodak) in Phosphate Buffered Saline at 37°C for 60min.

Step 8: The immobilised products are denatured in 0.1M sodium hydroxide at ambient temperature for 15 min.

Step 9: The wells are washed with 6 x SSC to remove the non-biotinylated strand. (6 x SSC is prepared by appropriately diluting 20 x SSC; 20 x SSC = 3M sodium chloride, 0.3M sodium citrate, pH 7.0)

5 **Step 10:** The appropriate ARMS primer (Table 13), (1 μ m) in 6 x SSC is hybridised to the immobilised single strand at 60°C for 60min then the wells are washed with 6 x SSC.

Step 11: The ARMS primer is extended in 50 μ l 0.1 mM dNTPs, 5 μ M digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (Boehringer Mannheim) with 2 units AmpliTaq™ DNA
10 polymerase (P. E. Applied Biosystems) in 1 x ARMS buffer at 60°C for 10 min.

Step 12: The extension reaction is terminated by the addition of 100 μ l 0.5M EDTA and the wells are washed with 25mM Tris-HCl (pH 7.5), 125 mM NaCl, 0.3mM MgCl₂ 0.3% Tween-20 (TBST).
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Step 13: Alkaline phosphatase conjugated anti- digoxigenin antibody (Boehringer Mannheim) is bound to extension products from the ARMS primers in TBST at 37°C for 30 min. then wells are washed with 1M diethanolamine (pH 9.8).

20 **Step 14:** p-Nitrophenol phosphate (Concn 1mg/ml) in 1M diethanolamine (pH 9.8) is added and incubated at ambient temperature for 30min.

Step 15: The optical density for each microplate microwell is measured at 405nm.

25 **Example 3**

Identification of k12 *ras* mutations by arms primer extension in combination with "Taqman"™

Table 16: Final concentrations of reagents

Reagents are prepared according to the table in 45µl aqueous solution

Reagent	Final concentration
ARMS primer	1.0µM
Common primer	1.0µM
dNTPs	100µM
ROX standard ^b	60nM
Reagent	Final concentration
TaqMan probe	200nM
MgCl ₂ ^c	3.5mM
Template DNA	Approx 10 ⁵ copies
ARMS buffer	1.0X ^a

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^a1X ARMS buffer = 10mM Tris-HCl, (pH 8.3) 1.2mM MgCl₂, 50mM KCl, 0.01% gelatin.

^bROX = 6-carboxyrhodamine. ^cIn addition to contribution from ARMS buffer.

Tables 17a and 17b: ARMS primer, common primer and "TaqMan"TM probe sequences

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Table 17a: Probe and primer sequences according to individual test

Probe/Primer	Sequence (5' - 3')
"TaqMan" [®] probe ^a	CAAGAGTGCCTTGACGATACAGCTA
Common primer (cpB)	CTCATGAAAATGGTCAGAGAAACCTTTATC
K12 CYSTEINE(ARMS)	CTGAATATAAACTTGTGGTAGTTGGAGCAT
K12 SERINE(ARMS)	CTGAATATAAACTTGTGGTAGTTGGAGCCA
K12 ARGinine(ARMS)	CTGAATATAAACTTGTGGTAGTTGGAGCCC

Table 17b: Probe and primer sequences according to individual test

Probe/Primer	Sequence (5' - 3')
"TaqMan" TM probe ^a	CTACCACAAGTTTATATTCAGTCATTTTCA
Common primer (cpA)	GTACTGGTGGAGTATTTGATAGTGTATTAACC
K12 VALINE(ARMS)	TATCGTCAAGGCACTCTTGCCTACGCCTA
K12 ASPARTATE(ARMS)	TATCGTCAAGGCACTCTTGCCTACGCCTT
K12 ALANINE(ARMS)	TATCGTCAAGGCACTCTTGCCTACGCCTG
K13 ASPARTATE(ARMS)	CGTGTATCGTCAAGGCACTCTTGCCTACCT

^aTaqManTM (Holland PM, et al., Proc Natl Acad Sci USA 1991; 88: 7276-7280.) probes were prepared by (P.E. Applied Biosystems) with FAM reporter dye (5') and TAMRA quencher dye (3'), [FAM = 6-carboxyfluorescein; TAMRA = N,N,N',N'-tetramethyl-6-carboxyrhodamine]

"AmpliTaq Gold"TM (P. E. Applied Biosystems), 5µl (diluted to 400 units/ml in 1X ARMS buffer) is added.

10

Thermal cycling conditions for all tests are: 94°C, 20 min; followed by 50 cycles of 94°C, 41 sec; 60°C, 42 sec; 72°C, 52 sec.

Diagnostic fluorescence signals are measured using, for example, a 7700 Sequence Detector (P. E. Applied Biosystems).

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Example 4

Identification of K-*ras* mutations by ARMS Linear Extension coupled with SERRS

Steps 1-6.

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Step 1: K-*ras* exon 1 is isolated from other non-K-*ras* human DNA sequences by hybridisation to a suitable immobilised capture sequence. An example of a suitable capture sequence is: 3'-ATTTCCAAAGAGACTGGTAAAAGT-5', however, other

suitable capture sequences, known to persons skilled in the art, may be used. The capture probe is immobilised to a suitable support so that non-hybridised sequences remain free in solution and are easily removed. The capture sequence may be bound to the support by any convenient method. For example, the sequence may be biotinylated at its 5' end and bound to a support coated in avidin/streptavidin. Hybridisation conditions may employ any suitable combinations of time, temperature and buffer constituents, determined by consideration of the T_m of the probe/*K-ras* target complex.

Capture of *K-ras* DNA may be achieved in a single hybridisation step. Alternatively, repeated rounds of hybridisation may be used; in this way the total amount of human genomic DNA hybridised is reduced but the relative level of *K-ras* to non- *K-ras* DNA bound after hybridisation, and removal of non-hybridised DNA, is greatly increased.

Step 2: Hybridised DNA may be eluted from the capture probe sequence using any convenient combination of temperature and/or buffer. Alternately if the capture probe sequence does not overlap with the site of prospective mutation analysis, mutation analysis by ARMS extension may be performed *in situ* i.e. directly on the immobilised *K-ras* sequence.

Step 3: The appropriate ARMS primer (Table 13), (1 μ m) in 6 x SSC is hybridised to the immobilised single strand at 60°C for 60min then the wells are washed with 6 x SSC.

Step 4: The ARMS primer is extended in 50 μ l 0.1 mM dNTPs with 2 units AmpliTaq™ DNA polymerase (P. E. Applied Biosystems) in 1 x ARMS buffer at 60°C for 10 min.

Step 5: The extension reaction is terminated by the addition of 100 μ l 0.5M EDTA and the wells are washed with 25mM Tris-HCl (pH 7.5), 125 mM NaCl, 0.3mM MgCl₂ 0.3% Tween-20 (TBST).

Step 6: Extended *K-ras* primers may be detected by any suitable detection method such as

surface enhanced ramon resonance spectroscopy (SERRS) whereby the extension product of an ARMS primer is hybridised to a complimentary sequence localised with a silver (AG) coated support. Hybridisation is detected by the change in SERRS signal measurable on hybridisation.

5

Example 5

System for the homogeneous detection of K-*ras* mutant sequences using ARMS coupled with amplicon detection by means of Taqman or Molecular Beacons.

10 **Steps 1-2.**

Step 1: Reaction conditions are as described in Example 3. Amplicons may be detected using the “Taqman”™ probes shown in tables 17a and 17b. Alternatively, amplicons may be detected using the 2'-o-methyl-RNA Molecular Beacon probe sequences; 5' CGC GGU GCC UUG ACG AUA CAG CUA AUU CAG AAC GCG 3' and 5' CGC GGU GCU GAA AAU GAC UGA AUA UAA ACU UGU GGA CGC G 3' at 100nM final test concentration.

15

Using the 2'-o-methyl-RNA Molecular Beacon described above the following data sets are derived:

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a) Real-time amplification plots are generated using templates comprised of either 10^3 , 10^4 or 10^5 human diploid genome equivalents of DNA. Templates consist wholly of wild-type sequence or of a mixture of a single mutant species mixed with wild-type DNA in a ratio of 500 copies of wild-type to 1 copy of mutant. The mutant DNA is typically obtained from one of the cell lines described in Table 4.

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b) Reactions are replicated, by independent operators on different occasions, such that a final data set comprising up to 12 independent data points for each input DNA concentration and wild-type/mutant mix is obtained.

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c) An independent measurement of the concentration of DNA added is carried out in each case. This consists of a parallel real-time amplification of an amount (volume) of added DNA which is equal to that used in the ARMS reactions. The parallel amplification reaction acts as an independent verification of the initial DNA quantification carried out for the preparation of template mixes.

Step 2: The data sets generated in Step 1 are used to plot the control data graphs shown in Figure 5.

Example 6

Application of the real-time system for K-ras mutant sequence detection described in Example 5.

Step 1: Reaction mixes described in Example 3 containing either the "Taqman"TM probe sequences described in Example 3 or the Molecular Beacon sequences described in Example 5 are prepared and dispensed into a 96 well plate as outlined in Figure 6.

Step 2: 5 μ l volumes of purified DNA solution, derived from a source of clinical material, typically ranging from 10^3 to 10^5 total copy number/5 μ l are added to each well of a single row in a 96 well plate as outlined in Figure 6. Amplification is carried out using appropriate conditions such as those described in Example 3

Step 3: Each row of a 96 well plate will produce a data set derived from a single DNA source. Interpretation of the data may conveniently be made with reference to the data sets, or similar data interpretations systems, described in Figure 5. Where initial DNA concentration falls below the desired range of 10^3 to 10^5 a preliminary amplification reaction of limited cycle number may be performed and the amplicons thus generated used in place of pure genomic DNA. Alternatively, where the initial DNA concentration is greater than 10^5 (estimable from the control reaction) an appropriate dilution from the neat sample may be performed followed by repeat analyses. In some

circumstances it may desirable to perform a set of 8 reactions on two independent dilutions of the clinical sample derived DNA. This may be useful in situations where the sample contains impurities which may adversely affect the efficiency of the given ARMS reaction. However, since the control reaction itself comprises a mis-matched primer coupled with a common primer it is likely to be similarly affected and thus control for the presence of impurities.

Step 4: By analysing the results obtained, studies of the clinical relevance of the detection of *K-ras* mutations may be performed.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5 (i) APPLICANT:

(A) NAME: Zeneca Limited

(B) STREET: 15 Stanhope Gate

(C) CITY: London

(D) STATE: England

10 (E) COUNTRY: United Kingdom

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15

(ii) TITLE OF INVENTION: Assay

(iii) NUMBER OF SEQUENCES: 25

20 (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

25

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: GB 9715034.6

(B) FILING DATE: 18-JUL-1997

30

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

5 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TATCGTCAAG GCACTCTTGC CTACGCCTA

29

15 (2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

20 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CTGAATATAA ACTTGTGGTA GTTGGAGCAT

30

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

5 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CTGAATATAA ACTTGTGGTA GTTGGAGCCA

30

15 (2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

20 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CTGAATATAA ACTTGTGGTA GTTGGAGCCC

30

30

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

5 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TATCGTCAAG GCACTCTTGC CTACGCCTT

29

15 (2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

20 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TATCGTCAAG GCACTCTTGC CTACGCCTG

29

30

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

5 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CGTGTATCGT CAAGGCACTC TTGCCTACCT

30

15 (2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleic acid

20 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GTACTGGTGG AGTATTTGAT AGTGTATTAA CC

32

30

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- 5 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CTCATGAAAA TGGTCAGAGA AACCTTTATC

30

15 (2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- 20 (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GGGCCTCAGT CCCAACATGG CTAAGAGGTG

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(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CCCACCTTCC CCTCTCTCCA GGCAAATGGG 30

15 (2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

TATATGTGCC ATGGGGCCTG TGCAAGGAAG 30

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

5 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CTCCTACACC CAGCCATTTT TGGC

24

15 (2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 base pairs

(B) TYPE: nucleic acid

20 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CTGGATCTAG ACTCATGAAA ATGGTCAGAG AAACCTTTAT C

41

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(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CCTCGGAATT CGTACTGGTG GAGTATTTGA TAGTGTATTA ACC

43

15 (2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GTTTTCCCAG TCACGAC

17

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CAGGAAACAG CTATGAC

17

15 (2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CCTCTATTGT TGGATCATAT TCGTCCA

27

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(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

5 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

ACTCATGAAA ATGGTCAGAG AAACCTTTAT C

31

15 (2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

20 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CAAGAGTGCC TTGACGATAC AGCTA

25

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(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CTACCACAAG TTTATATTCA GTCATTTTCA

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15 (2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

TGAAAATGGT CAGAGAAACC TTTA

24

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(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CGCGGUGCCU UGACGAUACA GCUAAUUCAG AACGCG

36

15 (2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CGCGGUGCUG AAAAUGACUG AAUAUAAACU UGUGGACGCG

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(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- 5 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

GGAGCTGGTG GCGTAGGC

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CLAIMS

1. A diagnostic method for the detection of *K-ras* mutations in cancer, which method comprises contacting a test sample of nucleic acid with a diagnostic primer for a *K-ras* mutation in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when a *K-ras* mutation is present in the sample; and detecting the presence or absence of a diagnostic primer extension product.
2. A diagnostic method as claimed in claim 1 wherein the *K-ras* mutations detected comprise one or more of the following mutations:
 - K-ras* codon 12 GGC→AGT (glycine → serine)
 - K-ras* codon 12 GGT→CGT (glycine → arginine)
 - K-ras* codon 12 GGT→TGT (glycine → cysteine)
 - K-ras* codon 12 GGT→GAT (glycine → aspartic acid)
 - K-ras* codon 12 GGT→GCT (glycine → alanine)
 - K-ras* codon 12 GGT→GTT (glycine → valine)
 - K-ras* codon 13 GGC→GAC (glycine → aspartic acid)
3. A diagnostic method as claimed in claim 1 and claim 2 wherein the diagnostic primer for a *K-ras* mutation is used with a further amplification primer in one or more cycles of PCR amplification.
4. A diagnostic method as claimed in any one of claims 1-3 wherein the test sample of nucleic acid is firstly amplified.
5. A diagnostic method as claimed in claim 1 wherein the diagnostic primer extension product is detected using a non-isotopic labelling method or real-time detection.
6. A diagnostic method as claimed in claim 1 wherein two or more diagnostic primers are used in the same reaction vessel.

7. Diagnostic primers for the detection of *K-ras* mutations comprising the sequences given below which detect the following mutations respectively:

	CTGAATATAAACTTGTGGTAGTTGGAGCCA	K12 Serine
	CTGAATATAAACTTGTGGTAGTTGGAGCCC	K12 Arginine
5	CTGAATATAAACTTGTGGTAGTTGGAGCAT	K12 Cysteine
	TATCGTCAAGGCACTCTTGCCTACGCCTT	K12 Aspartic acid
	TATCGTCAAGGCACTCTTGCCTACGCCTG	K12 Alanine
	TATCGTCAAGGCACTCTTGCCTACGCCTA	K12 Valine
10	CGTGTATCGTCAAGGCACTCTTGCCTACCT	K13 Aspartic acid

8. A diagnostic kit comprising one or more of the diagnostic primers as claimed in claim 7, nucleotide triphosphates, polymerase, buffer solution and instructions for use.
9. A diagnostic kit as claimed in claim 8 comprising all seven diagnostic primers.



Application No: GB 9815224.2
Claims searched: 1-9

Examiner: Dr Jon Broughton
Date of search: 16 October 1998

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK CI (Ed.P):

Int CI (Ed.6): C12Q 1/68

Other: ONLINE: DIALOG/BIOTECH, WPI, DGENE, CAS ONLINE

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
X	GB 2299166 A (STROUN <i>et al.</i>) See whole document, particularly example 1 part b.	1-4 and 7.
X	WO 97/19191 A1 (ØGREID <i>et al.</i>) See page 21 line 10 - page 23 line 25.	1-4, 7 and 8.
X	WO 96/40995 A1 (TRUSTEES OF DARTMOUTH COLLEGE) See whole document.	1-4, 5, 7 and 8.
X	WO 91/13075 A2 (ORION-YHTYMÄ) See example 8.	1, 2, 4, 6 and 7.
X	US 5137806 (LeMAISTRE) See example 1.	1-4 and 7.
X	Oncogene (1991) 6 857-862 (STORK P. <i>et al.</i>) See whole document.	1-3, 7 and 8.

X Document indicating lack of novelty or inventive step
Y Document indicating lack of inventive step if combined with one or more other documents of same category.
& Member of the same patent family

A Document indicating technological background and/or state of the art.
P Document published on or after the declared priority date but before the filing date of this invention.
E Patent document published on or after, but with priority date earlier than, the filing date of this application.